A TEM and EPR Investigation of the Competitive Binding of Uranyl Ions to Starburst Dendrimers and Liposomes: Potential Use of Dendrimers as Uranyl Ion Sponges

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Transmission electron micrographs (TEM) of UO_2^{2+}-negatively stained starburst dendrimers (SBDs), members of the family of dendritic macromolecules, have been analyzed in the absence and in the presence of dimyristoyl-phosphatidylcoline (DMPC) liposomes and mixed DMPC/DMPA-Na (the sodium salt of DMP-colate) liposomes at different relative percentages of DMPC and DMPA-Na. Under most conditions with dendrimers present, the dendrimers, rather than the liposomes, are visible in the TEM images, demonstrating that the UO_2^{2+} is complexed to the dendrimers and not to the liposomes. Only at high composition of DMPA-Na in the liposomes (>40%) and under the condition of high protonation of the dendrimer surface are the liposomes imaged by TEM. Mixed liposomes show a rodlike shape. To confirm the TEM results, an EPR study was performed by adding to the SBD solution various amounts of Cu^{2+} and UO_2^{2+}. Uranyl ions compete favorably with copper ions for the complexation with the nitrogen ligand sites at both the external and the internal dendrimer surfaces. The saturation of the dendrimer by Cu(II) occurs at about 33% complexation of the nitrogen groups. The stability constant of the Cu^{2+}−SBD complex was evaluated, along with an indirect estimation of the stability of the UO_2^{2+}−SBD complex. The results demonstrate that starburst dendrimers selectively bind to uranyl ions and that the latter compete effectively for these sites, even with Cu(II) ions, which are well-known for forming stable complexes with nitrogen ligands. These results show that dendrimers have the potential for storing uranium derivatives, a process of great importance in the fields of energy production and environmental cleanup.

Introduction

The problem of extraction and storage of radioactive heavy-atom derivatives by selective complexation with ligand groups is an area of current environmental interest and importance.[1] Among the uranium compounds, uranyl ions (UO_2^{2+}) show a low preference for complexation by nitrogen ligands.[2] Polyamidoamine starburst dendrimers (SBDs),[3] members of the family of dendritic macromolecules,[4] provide a high concentration of nitrogen ligands per dendrimer molecule on the internal and external surface. If binding of UO_2^{2+} to dendrimers can be made efficient, dendrimers could serve as uranyl ion "sponges" of potential use in environmental clean ups. We report an investigation by TEM and EPR of the complexation of UO_2^{2+} to dendrimers, which demonstrates an unexpectedly efficient binding.

Recently, SBDs have been investigated for their promising use as efficient vectors for gene transfection in mammalian cells.[5,6] To provide insight into the mechanism of dendrimer-assisted transfection, electron paramagnetic resonance (EPR) studies[7−10] have been employed to investigate several binary systems consisting of SBDs and micelles or liposomes[11−13] as membrane models for the biosystems.

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Liposomes composed of dimyristoyl-phosphatidylcoline (DMPC) have been characterized by transmission electron micrographs (TEM) in both the presence and the absence of dendrimers employing the negative staining technique,[7,15] using uranyl acetate as the staining agent.

Although TEM has been successfully used to characterize both dendrimers and liposomes,[7,16,17] *TEM measurements showed the disappearance of negatively stained (UO_2^{2+}) vesicles in the presence of the dendrimers*. To investigate the origin of this observation, we performed TEM studies, with UO_2^{2+} as the contrast agent, on mixtures of SBDs and vesicles composed of DMPC and different percentages of the negatively charged phosphate-sodium salt of DMPC (DMPA-Na), by varying the concentrations of the dendrimers and extent of protonation of the dendrimer surface.[18] The variation in composition of the liposomes, that is, the relative concentrations of DMPC and DMPA-Na, not only changes the electrostatic interactions of the liposomes with charged ions and surfaces but also[19] modifies the aggregation behavior of the surfactants, increasing the aggregation number and modifying the shape and size of the liposomes.

Further information on the interactions between the uranyl ions and the dendrimers was obtained by an indirect spectroscopic method. Cu^{2+} has recently been shown by EPR analysis to form very stable complexes with the nitrogen sites at the internal and external dendrimer surface.[20,21] Therefore, EPR provides a means of monitoring the competition between Cu^{2+} and UO_2^{2+} in the complexation with the nitrogen centers at the dendrimer surface. EPR analysis also allows an evaluation of the stability constants of the complexes of dendrimers with the two ions.

Two dendrimers were studied, 6SBD and 2SBD (6 and 2 are the numbers of monomeric layers, termed generations). However, because the results from the smaller dendrimer were comparable to those from the larger dendrimers, we report and discuss below the results only for 6SBD (256 surface groups).

Experimental Section

Liposomes composed of varying percentages of DMPC and DMPA-Na (0%, 20%, and 40%) were prepared[14] by swelling the dried lipid film in a sucrose solution without shaking, followed by double spinning intercalated by the addition of a glucose solution. The resulting pellet was stored in a refrigerator under nitrogen and dissolved in micropore water to provide a maximum concentration of 1×10^{-9} M in liposomes.

TEM experiments were carried out using the negative staining technique[7,15] employing 2 wt %/wt uranyl acetate as the staining agent. The samples were stained for 2 h after adsorption onto a nickel net which was covered by a Formvar (polyvinyl−formaldehyde) film. After washing out the excess unbound UO_2^{2+}, TEM measurements were obtained under vacuum by means of PHILIPS-EM 201-TEM instrumentation. The concentration of SBD was varied in the range of 5×10^{-2}−5×10^{-5} M in external surface groups at a constant concentration of liposomes (about 10^{-10} M). The level of protonation of the dendrimers was varied by the addition of dilute HCl solutions to the samples. The pH of an aqueous solution of native 6SBD is ca. 8−9, corresponding to about 70% protonation of the amino groups at the external surface. For aqueous solutions of 6SBD acidified to pH = 4−5, the dendrimer is a highly protonated dendrimer (all the external and some of the internal amino groups are protonated).[18]

EPR spectra were recorded by means of a Bruker 200D spectrometer operating at X band (9.5 GHz) and interfaced with Stelar software to a PC-IBM computer for data acquisition and handling. Temperature was controlled with a Bruker ST 100/700 variable temperature assembly. The EPR spectra were recorded at 298 K. The magnetic parameters were measured by field calibration using diphenylpicrylhydrazyl (DPPH, g = 2.0036). Unless otherwise specified, the spectra were obtained by adding a variable number of scans, reported in the figure captions, at a speed of 120 s each (gain 1×10^4 and field modulation of 3 G). The samples for EPR were prepared by mixing a $Cu(NO_3)_2$ (Merck)−water (Millipore-purified) solution (final concentration 0.01 M) with a 6SBD−water solution (final concentration of 0.04 M in amino surface groups) and then adding the uranyl acetate solution at different concentrations (final concentrations 0.005−0.05 M). Addition of the three components either in a different sequence (e.g., mixing dendimer with uranyl ions, and then adding copper ions) or keeping the UO_2^{2+} concentration constant and changing both the Cu^{2+} and the SBD concentrations did not modify the quantitative results. Solutions of 0.04 M SBD with increasing amounts of Cu^{2+} (up to 0.5 M) in the absence of uranyl ions were also analyzed to measure the saturation of the dendrimer interacting sites by the copper ions. As with the TEM samples, good reproducibility was observed for the EPR samples.

Results and Discussion

Figure 1 shows three representative TEMs of liposome−dendrimer samples under staining with uranyl ions; Panels a and b are at the same magnification (1 cm = 0.045 μm), and panel c is at a lower magnification of 1 cm = 0.89 μm.

Figure 1a shows a TEM of DMPC liposomes + 6SBDs at a concentration of 0.05 M in the surface amino groups. The same features are observed at SBD concentrations as low as 5×10^{-5} M and for mixed liposomes (DMPC + DMPA-Na) with 0.05 M SBDs. Figure 1b shows a TEM of liposomes containing a mixture of DMPC and DMPA-Na (40%) + 6SBDs at a concentration of 5×10^{-5} M in the surface amino groups. Figure 1c shows a TEM of liposomes containing a mixture of DMPC and DMPA-Na (40%) + 6SBDs under conditions of high protonation (pH = 4.5) at a concentration of 5×10^{-5} M in the surface amino groups**.** In the absence of dendrimers, the micrograph of the liposomes is essentially the same as in Figure 1c except for the image of dendrimers which appear as small sandlike granules in the background of Figure 1c.

At a higher SBD concentration (0.05 M), the liposomes are not visible, even under the conditions of high protonation of the dendrimer surface and in the presence of 40% DMPA-Na in the liposomes. The small black spots in the TEMs are images of dendrimers both as isolated molecules and in small and large aggregates (the aggregation is probably favored by the collapse of the dendrimer molecules onto the vesicles). The size of each black spot roughly corresponds to the size of 6SBD (about 7 nm). An important observation is that at a lower SBD concentration (5×10^{-5} M), DMPC/DMPA-Na (40%) liposomes reappear as dark shadows in the presence of natively protonated (70% of surface amino groups at pH = 8−9) SBDs in Figure 1b and as dark spots with black external margins in the presence of highly protonated SBDs in Figure 1c. It is interesting to note that in the

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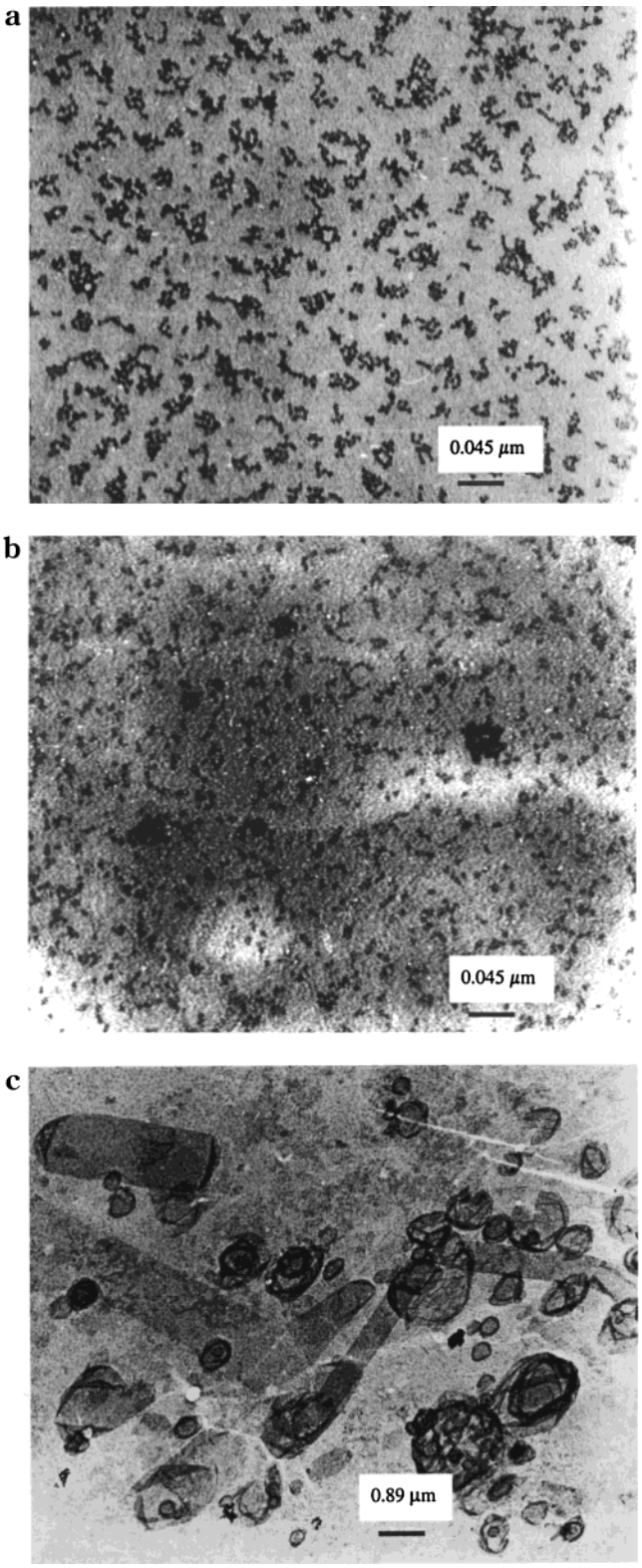

Figure 1. Representative TEMs of UO_2^{2+}-negatively stained liposomes of (a) DMPC liposomes + SBDs at generation 6 (6SBDs) at a concentration of 0.05 M in the surface amino groups. The same micrograph was obtained at SBD concentrations as low as 5×10^{-5} M and for mixed liposomes (DMPC + DMPA-Na) with 0.05 M SBDs. Magnification × 232 000 (1 cm = 0.045 μm). (b) DMPC/DMPA-Na (40%) liposomes + 6SBDs at a concentration of 5×10^{-5} M in surface amino groups. Magnification × 232 000 (1 cm = 0.045 μm). (c) DMPC/DMPA-Na (40%) liposomes + 6SBDs highly protonated at a concentration of 5×10^{-5} M in surface amino groups. Magnification × 11 200 (1 cm = 0.89 μm). (Figure was reduced to 60% of original for publication.)

latter case, the liposomes show two different shapes: *spherical*, with an average size between 0.5 and 1.5 μm, found for pure DMPC liposomes,[7] and large *cylindrical*-shaped liposomes with an average size of ca. 1.5 × 6 μm, which form only in the presence of DMPA-Na. Indeed, the relative number of these cylindrical features decreases with a decrease in the percentage of DMPA-Na. Differently shaped and sized liposomes were expected on the basis of previous studies[19] for mixed neutral + charged phospholipid samples.

On the basis of the TEM results and previous studies,[7] we propose the following:

(1) The disappearance of the liposomes' images in the presence of the dendrimers cannot be ascribed to the fragmentation and disruption of DMPC vesicles due to their interaction with the dendrimers, because previous studies[7,9] demonstrated that these vesicles are not destroyed by the dendrimers. Furthermore, a dilution of the DMPC/DMPA-Na + 6SBD solutions shows a progressive reappearance of the intact liposomes in the micrographs (as dark shadows of the same size and shape as in Figure 1b,c).

(2) *The uranyl ions selectively interact and bind with the dendrimer surface (the external amino groups and also, on the basis of the EPR results shown below, the internal nitrogen sites) so that the uranyl contrast agent is transferred from the liposomes to the dendrimers.* As a result, the liposomes become "invisible" (not imaged) by TEM analysis. On the basis of literature results,[2] which claim that UO_2^{2+} rarely binds strongly to nitrogen ligands, this conclusion was unexpected. However, examples of the binding of uranyl ions with nitrogen ligands in polymeric materials have been reported.[22,23]

(3) A concentration of dendrimers as low as 5×10^{-5} M (in surface amino sites) is sufficient to sequester the uranyl ions from the solution, preventing their interaction with the liposome surface. The external surface 6SBD consists of 256 primary amino groups and 756 internal nitrogen centers, both tertiary amino and amido groups. Therefore, the true concentration of possible nitrogen binding sites is about 2×10^{-4} M. If we assume a 1:4 complexation between UO_2^{2+} and nitrogen sites, as is usually found for such complexes, the maximum concentration of UO_2^{2+} complexed with the dendrimers at this concentration is 5×10^{-5} M. However, we cannot exclude a complexation between hydrolyzed uranyl ions and the dendrimer surface groups.

(4) Even a partially negatively charged surface of the liposomes (40% of DMPA-Na) does not compete favorably with a low concentration of dendrimers for binding UO_2^{2+}.

(5) The essentially complete reappearance of the liposomes in the micrographs is observed when the conditions of (a) high protonation of dendrimers (pH = 4.5), (b) low concentration of dendrimers, and (c) presence of the negatively charged DMPA−Na are met simultaneously. This is expected because the protonated, low-concentration dendrimer competes inefficiently with the liposome surface for complexation of the uranyl ions.

(6) The observation that the mixed DMPC/DMPA-Na liposomes show two different shapes, spherical and cylindrical, leads to the conclusion that the negatively charged phospholipid does not homogeneously distribute in the liposomes, and in the case of surface regions mainly constituted by these negative groups, the charge repulsion

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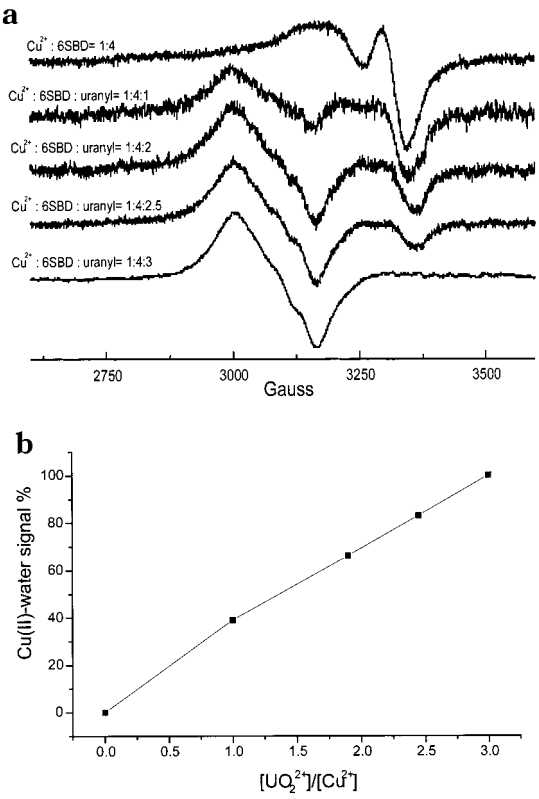

Figure 2. (a) EPR spectra (298 K) obtained for a 0.01 M water (Millipore-filtered) solution of $Cu(NO_3)_2$ and 0.04 M (in surface amino groups) solution of 6SBD in the absence and in the presence of increasing amounts of uranyl acetate, i.e., 0.01, 0.02, 0.025, and 0.03 M. The number of scans used for recording the EPR spectra was 5, 2, 3, 5, and 8 from top to bottom, respectively. (b) Variation of the percentage of relative intensity of the Cu−water signal as a function of the ratio between the uranyl and the Cu(II) concentrations.

favors the formation of cylindrically shaped liposomes.[19] However, the spherical liposomes reappear in the micrographs, indicating that the presence of DMPA-Na in the liposomes is not required to remove UO_2^{2+} from the dendrimer surface under conditions of high protonation and low concentration of the dendrimers themselves.

(7) The dendrimers randomly distribute both at the liposome surface and in the spaces among the liposomes. However, the micrograph in Figure 1b shows a higher concentration of dendrimer balls, mainly as aggregates, corresponding to the shadows due to the liposomes.

To provide independent support to the important conclusion that the uranyl ions interact selectively with the dendrimer amino surface groups rather than the surface of the liposomes, an EPR study was performed employing a different, experimentally established competitor to uranyl ions for dendrimer complexation. Cu^{2+} has been shown to form very stable complexes with the amino groups at the dendrimer surface.[20,21] In this case, highly protonated dendrimers do not show direct complexation of Cu^{2+} with the internal amino groups. Therefore, only partially protonated dendrimers (70% of the surface groups at pH = 8−9) were used in the EPR study. Figure 2a shows some representative EPR spectra (298 K) obtained for a 0.01 M water solution of $Cu(NO_3)_2$ and 0.04 M in surface amino groups of 6SBD in the absence and in the presence of increasing amounts of uranyl acetate (0.01, 0.02, 0.025, and 0.03 M, from top to bottom, respectively). The signal in the absence of UO_2^{2+} is characteristic of Cu^{2+} complexed by internal nitrogen centers in the dendrimer structure under slow-motion conditions (correlation time for the rotational motion evaluated from computation by means of the program of Schneider and Freed:[24] $\tau = 1.5 \times 10^{-9}$ s, whereas the magnetic parameters are $\langle g \rangle = 2.10$ and $\langle A \rangle = 67$ G) due to the trapping in the internal structure.[20] The magnetic parameters evaluated by computation (program CU23)[25] of the spectrum at low temperature (result not shown) are $g_{xx} = 2.03$, $g_{yy} = 2.06$, $g_{zz} = 2.21$; $A_{xx} = 9.5 \times 10^{-4}$ cm^{-1}, $A_{yy} = 1.2 \times 10^{-4}$ cm^{-1}, $A_{zz} = 184 \times 10^{-4}$ cm^{-1}. These parameters are indicative of Cu^{2+} complexes in an almost square planar configuration with $d_{x^2-y^2}$ as ground state, where copper ions coordinate to four nitrogen centers. Therefore, Cu^{2+} is inserted into the dendrimer structure interacting with 4-N sites. The addition of UO_2^{2+} gives rise to a new signal at lower field ($\langle g \rangle = 2.21$ and $\langle A \rangle =$ 44 G, as obtained from computation of the line shape),[24] characteristic of Cu(II) in water solution. The relative intensity (percentage) of the Cu−water signal was obtained by subtraction of each signal from the overall line shape and double integration of the obtained components. This Cu−water signal increases its relative intensity at the expense of the Cu−dendrimer signal, with the increase of the ratio between the uranyl and the Cu(II) concentrations, as shown in Figure 2b. An almost linear relationship is observed. It is clear from Figure 2 that UO_2^{2+} and Cu^{2+} compete to occupy the nitrogen ligand sites at the dendrimer surface so that at a 1/1 ratio, about 40% of the complexing sites are exchanged by UO_2^{2+}, and that is enough for a ratio of 3/1 to completely extrude Cu^{2+} from the dendrimer structure. The stability constant of the UO_2^{2+}−SBD complex may be roughly computed on the basis of these results and a comparison with the formation and stability of the Cu−SBD complex. The titration of the adsorption capability of the dendrimer toward Cu(II) is obtained by recording the EPR spectra as a function of the [SBD]/[Cu] ratio. EPR spectra were recorded and analyzed either by keeping the Cu(II) concentration constant and varying the 6SBD concentration, or by keeping the SBD concentration constant and varying the Cu(II) concentration. The results demonstrated that the true variable is the [SBD]/[Cu] ratio, with no dependence being found on the different titration procedures or different fixed concentration. The insert in Figure 3 shows the series of EPR spectra obtained at various Cu/SBD ratios. Obviously, in the absence of the dendrimer (Cu/SBD = 1/0), only the signal of Cu(II) in water is present. By increasing the [SBD]/[Cu^{2+}] ratio to 3, the Cu−SBD signal almost linearly increases at the expense of the Cu−water signal. Then a minimum percentage (<10%) of the Cu−water signal remains present up to [SBD]/[Cu] = 6. Only at [SBD]/[Cu^{2+}] =10 is the Cu−water component absent from the EPR signal. The graph of the relative percentage of the signal of noninteracting Cu(II) (spectrum of aqueous Cu^{2+} complex) as a function of the Cu(II) concentration is also shown in Figure 3. The extrapolation of the point at which the linear increase of the Cu−water component begins provides a value of [SBD]/[Cu] = 3.2, which indicates the saturation of the interacting sites of the SBD by means of the complexed Cu^{2+}. This ratio corresponds to [Cu(II)] = 0.0125 M for [SBD] = 0.04 M. Assuming a minimum of 5% of the 0.0125 M of Cu(II) free

(24) Schneider, D. J.; Freed, J. H. In *Biological Magnetic Resonance. Spin Labeling. Theory and Applications*; Berliner, L. J., Reuben, J., Eds.; Plenum Press: New York, 1989; Vol.8, p 1. This program was primarily created for computation of EPR spectra of nitroxide radicals under slow-motion conditions, but it has also been successfully used for computing the EPR spectra of Cu^{2+} under slow-motion conditions.[20]

(25) Program for computation of the EPR spectra of Cu^{2+} in frozen solutions was kindly provided by Prof. M. Romanelli, University of Florence, Italy.

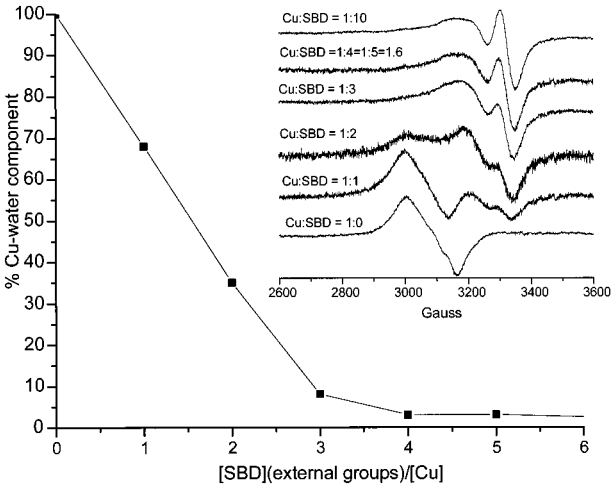

Figure 3. Variation of the percentage of relative intensity of the Cu−water signal as a function of the ratio between the SBD concentration, in external groups, and the Cu^{2+} concentration, in the absence of uranyl ions. The insert shows the experimental EPR spectra of 6SBD at concentration of 0.04 M and increasing amounts of Cu^{2+} (bottom spectrum, $[Cu^{2+}] =$ 0.01 M). The number of scans used for recording of the EPR spectra was 15, 5, 4, 2, 2, and 8, from top to bottom, respectively.

in solution at the saturation ($= 6.25 \times 10^{-4}$) and considering that the 0.04 M concentration in external amino groups corresponds to a 0.158 M concentration in total nitrogen sites, the stability constant for the reaction of complexation $Cu^{2+} + 4$ N (SBD sites) $= [CuN_4]^{2+}$ is

$$K = 0.012/\{6.25 \times 10^{-4} \times [0.158 - (0.012 \times 4)]^4\} = 1.3 \times 10^5\ M^{-1}$$

Also, an evaluation of the occupation of N-dendrimer sites from the linear increase in the Cu−water signal indicates that only about 30% of the nitrogen sites are involved in the complexation with Cu(II) (and uranyl, too), corresponding to about 80 Cu^{2+} mol for 1 mol of the SBD macromolecule. This result is consistent with the absence of spin−spin interactions among Cu^{2+} ions in the dendrimer structure; the saturation is reached when the Cu(II) ions are located in dendrimer cavities that are not adjacent.

Because a 3-fold excess of uranyl ions is required to replace the copper ions on the dendrimer surface, we conclude that the stability constant for the uranyl−dendrimer complexation is about 3 times lower than that found for copper, that is, about $4 \times 10^4\ M^{-1}$. Of course, this value only gives a rough value of the binding affinity of UO_2^{2+} for the dendrimer nitrogen sites.

Summary and Conclusions

TEMs of UO_2^{2+}-negatively stained 6SBDs and DMPC liposomes show only the images of dendrimers with the size expected (size of 6SBD, ca. 7 nm), whereas the liposomes seem to disappear, even under conditions which have established the liposomes' being present (light scattering and EPR).[7] Therefore, it is concluded that the dendrimers sequester the UO_2^{2+} staining agent from the solution, in competition with the complexing of uranyl with the liposomes. Only by adding DMPA-Na in the DMPC liposomes at amounts as large as 40% and on condition of high protonation of the dendrimer surface do the liposomes recover their visibility by TEM. Mixed liposomes showing a rodlike shape are observed under these conditions. To confirm the TEM results, an EPR study was performed by adding different relative amounts of Cu^{2+} and UO_2^{2+} to the SBD solution. Uranyl ions compete favorably with copper ions for the complexation with the nitrogen ligand sites at the external and internal dendrimer surface. To provide quantitative information on the binding strength of the Cu(II)−SBD complexes and indirectly on the SBD complexation by UO_2^{2+}, a titration of the SBD ligand groups was performed by increasing the Cu/SBD ratio in solution and analyzing the resulting EPR spectra. The saturation conditions showed that only about 30% of totally available nitrogen sites are involved in the complexation with Cu(II) and uranyl, corresponding to about 80 Cu^{2+} mol for 1 mol of SBD macromolecule. The stability constants of the Cu^{2+}−SBD and the UO_2^{2+}−SBD complexes are roughly evaluated as 1.3×10^5 and 4×10^4 M^{-1}, respectively. Therefore, starburst dendrimers provide selective binding sites to uranyl ions showing an effective competition even with Cu(II) ions, which are well-known to form stable complexes with nitrogen ligands.

These results are very promising for using dendrimers for storing uranium derivatives, a process of great importance in the fields of energy production and environmental cleanup. The dendrimers are very stable and constitute a potential protective shell for the uranyl ions trapped in their structure, which can allow the capture and inhibition of the diffusion of these ions in the environment.

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